Veterinary Parasitology 237 (2017) 110–116

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

The *in vitro* anthelmintic properties of browse plant species against *Haemonchus contortus* is determined by the polyphenol content and composition

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ARTICLE INFO

Article history:
Received 12 October 2016
Received in revised form
23 December 2016
Accepted 29 December 2016

Keywords:
Proanthocyanidins
Larvae
Exsheathment
Haemonchus contortus

ABSTRACT

The aims of the present study were to (a) evaluate the anthelmintic activity of 10 East African browse plant extracts, (b) examine their role in inhibition of *Haemonchus contortus* larval exsheathment, (c) establish relationship between inhibition of larval exsheathment and browse plant extract polyphenol composition. Acetone/water (70/30%) extracts of air dried leaves of *Acacia etbaica*, *Cadaba farinosa*, *Capparis tomentosa*, *Dichrostachys cinerea*, *Dodonaea angustifolia*, *Euclea racemosa*, *Maerua angolensis*, *Maytenus senegalensis*, *Rhus natalensis* and *Senna singueana* were used. The larval exsheathment inhibition assay (LEIA) was applied using *H. contortus* third stage larvae (L_3) and browse plant extract concentrations of 0, 150, 300, 600, 1200 μg/ml in phosphate buffered saline (PBS). Data were analysed using the PROC MIXED procedure of SAS. Polyvinylpolypyrrolidone (PVPP) was used to evaluate whether polyphenols were involved in L_3 exsheathment inhibition. All browse plant extracts significantly ($P \leq 0.001$) inhibited larval exsheathment in a dose dependent manner. The dose required to inhibit 50% of the larvae (EC_{50}) was highest in *C. farinosa* and lowest in *E. racemosa* and *M. senegalensis*. Significant differences ($P < 0.001$) between the control and PVPP treated *A. etbaica*, *C. tomentosa*, *M. angolensis*, *R. natalensis* and *D. cinerea* indicates that larval inhibition was largely due to non-phenol compounds. For *E. racemosa*, *M. senegalensis*, *D. angustifolia* and *S. singueana*, PVPP treatment reversed inhibition activity and in these extracts, inhibition was mostly attributable to tannin and other polyphenols (kaempferol, quercetin and myricetin based glycosides). Overall, the browse plant extracts have anthelmintic property against *H. contortus* and larval inhibition resulting from the presence of phenolic and non-phenolic compounds.

1. Introduction

Economic losses due to infections with gastrointestinal nematodes (GINs) represent a major challenge for small ruminant producers in outdoor grazing systems (FAO, 2002). Impaired productivity in infected goats and sheep results from reduction in voluntary feed intake with the associated decrease in nutrient availability, nutrient absorption and efficiency (Coop and Kyriazakis, 2001). Sheep and goats have important economic roles in tropical and subtropical climates (FAO, 2002; Jackson et al., 2012), and the combination of high temperature and rainfall in these areas provides a conducive environment for the development of the infective larval stage of nematodes (Waller, 1997). Until recently, the control of parasitic GINs in small ruminants has mainly been based on the quasi-exclusive reliance through the use of available synthetic, commercial drugs (Jackson et al., 2012; Oliveira et al., 2011). However, the rapid development of anthelmintic resistance, which is defined as the decline in heritable sensitivity of parasites upon drug application (Conder and Campbell, 1995) in parasite populations, is resulting in failure of the efficacy of these drugs (Jackson et al., 2012). *Haemonchus contortus* is the most commonly occurring resistant parasite due to its highly prolific nature, prompting establishment on pasture within a short time (Conder and Campbell, 1995; Waller and Chandrawathani, 2005). The use of anthelmintic

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http://dx.doi.org/10.1016/j.vetpar.2016.12.020
0304-4017/

drugs also raises public health concern (Oliveira et al., 2011). Moreover, in developing countries, the use of anthelmintic drugs may be limited due to inaccessibility or high cost (Debela et al., 2012). Forages possessing plant secondary metabolites (PSM) have potential use as alternative natural treatments used either as herbal drugs or nutraceuticals (Oliveira et al., 2011). Such an approach is of practical value under low input small ruminant production systems by providing a practical, sustainable (Sokerya and Preston, 2003; Max et al., 2007) and affordable (Salem and Smith, 2008) alternative control of GINs.

Forages containing condensed tannins (CT) are the most widely studied models as nutraceuticals, with combined beneficial effects on nutrition and health (Hoste et al., 2012, 2015). Previous *in vivo* and *in vitro* studies with a range of tannin-containing forages have demonstrated anthelmintic effects against *H. contortus* by reduction of larvae establishment (Brunet et al., 2008; Oliveira et al., 2011) and reduction in egg excretion in goats *e.g.* when given quebracho tannins (Paolini et al., 2003). These anthelmintic properties are attributable to the ability of PSM such as CT to boost host resilience and/or modulate nematode biology. Although CT were proven to have anthelmintic properties, their mode of action, which also seems to vary on the diverse nature of tannins depending on the source and chemical structure, still remain insufficiently identified.

CT or proanthocyanidins (PA) represent one of the common groups of tannins, and are oligomers and polymers of flavan-3-ol monomer units. PA could be grouped into six main classes (Salminen and Karonen, 2011). Procyanidins (PC) are the most common PA containing (+)-catechin with 2*R*,3*S* stereochemistry and (−)-epicatechin with 2*R*,3*R* stereochemistry as monomeric units; while prodelphinidins (PD) consist of gallocatechin and epigallocatechin monomeric units. Propelargonidins (PP) contain afzelechin and epiafzelechin, profisetinidins (PF) fisetinidols and epifisetinidols, prorobinetinidins (PR) robinetinidols and epirobinetinidols, and proguibourtinidins (PG) guibourtinidols and epiguibourtinidols. The PF, PR and PG, possess the rarer monomeric 5-deoxy units of CT in their structure (Salminen and Karonen, 2011). In addition to six main PA classes, some less common PA classes also exist. Some previous work reported the association between PA composition and anthelmintic properties. A marked reduction on *H. contortus* L_3 exsheathment *in vitro* has been associated with the PD monomers and with the galloyl derivatives of tannins (Brunet and Hoste, 2006). Quijada et al. (2015) reported that the building units of PA and the polymer size influenced anthelmintic properties in *in vitro* larval exsheathment of *H. contortus*.

Another important tannin group consists of hydrolysable tannins (HT) which are esters of gallic acid and a polyol, which in most cases is D-glucose. HT are divided into three subclasses, *i.e.* simple gallic acid derivatives, gallotannins and ellagitannins. Variability of HT in the size, type and monomeric unit linkages have also been associated with anthelmintic properties measured by the inhibition of egg hatching and larval motility of *H. contortus* (Engström et al., 2016). Moreover, the presence of co-occurring polyphenols or non-phenolic compounds in tannin-containing forages may contribute to the anthelmintic properties of plant extracts (Barrau et al., 2005; Azando et al., 2011; Azaizeh et al., 2013). Polyphenols of interest in anthelmintic studies other than tannins, may include, for example quinic acid derivatives, quercetin-based flavonol glycosides, kaempferol-based flavonol glycosides and myricetin-based flavonol glycosides. Therefore, for the efficient exploitation of polyphenolic composition of tannin-containing forages, the simultaneous analysis of PA concentration and composition; and the other phenolic compounds is required. This information allows comparisons of anthelmintic properties of polyphenols from different plant sources and across studies. The browse plant species in the present study grow in semi-arid areas (Bein et al., 1996) and are readily consumed by goats in these areas (Yayneshet et al., 2008; Mengistu et al., 2016). However, their anthelmintic properties against goat nematodes are unknown.

The objectives of the present work were (a) to evaluate the anthelmintic activity of a range of browse plant extracts from eastern Africa using an *in vitro* assay measuring the exsheathment of *H. contortus* L_3 larvae in a dose dependent manner, (b) to examine the role of tannins and other polyphenols in the inhibition of *H. contortus* exsheathment using a tannin binding agent, polyvinylpolypyrrolidone (PVPP), (c) to establish the relationship (if any) between browse plant polyphenol content and composition with the inhibition of L_3 exsheathment, so as to provide a model to better understand the anthelmintic activity in relation to polyphenol composition.

2. Materials and methods

2.1. Browse plant collection and handling

Leaves (Table 1) of *Acacia etbaica*, *Cadaba farinosa*, *Capparis tomentosa*, *Dichrostachys cinerea*, *Dodonaea angustifolia*, *Euclea racemosa*, *Maerua angolensis*, *Maytenus senegalensis*, *Rhus natalensis* and *Senna singueana* were collected by hand-clipping from semi-arid Tigray region of Ethiopia at the end of the long rainy season (July–September) in 2014. Immediately after collection leaves were air-dried under shade, stored and ground to pass a 1 mm sieve. Specimens of the browse plant species were mounted on a placard and sent to the national herbarium institute of Addis Ababa University, Ethiopia for identification.

2.2. Extraction procedure

Ground browse plant samples were extracted with 70/30% acetone/water with a browse plant sample/solvent proportion of 1:4 (w/v). The supernatant obtained by extraction was further treated with dichloromethane with a proportion of 1:3 (v/v) to remove lipids and chlorophyll, freeze dried and stored until use.

2.3. Chemical analysis

For chemical analysis, ground browse plant samples were freeze-dried (Christ beta 2–8 LD plus, Martin Christ, Germany) and ground into a fine powder (Retsch MM200, Sigma-Aldrich, US). For each browse plant, approximately 20 mg of sample was weighed in triplicate in Eppendorf tubes, vortexed with 1.4 ml of acetone/water (4:1, v/v) for 5 min and allowed to macerate at 4 °C overnight. Then the samples were extracted in a planar shaker for 3 h and centrifuged at 21,913*g* for 10 min. The residues were further extracted with 1.4 ml of acetone/water (4:1, v/v) for 3 h and centrifuged at 21,913*g* for 10 min. The extracts were combined and concentrated into the water phase and freeze-dried. The freeze-dried extracts were dissolved in 1 ml of water.

PA contents were analysed as previously described by Grabber et al. (2013). Total PA were analysed directly from the browse plant materials. Soluble PA were analysed from the browse plant extracts and the insoluble, *i.e.* bound, PA from the extraction residues. PA were quantified against an external PA standard (extracted from *Calluna vulgaris*) which had a PC/PD ratio of 99:1 and a mean degree of polymerization of 4.9.

PA composition was determined by an ultra-performance liquid chromatograph coupled to a photodiode array detector (UPLC-DAD, Acquity UPLC, Waters Corporation, Milford, MA, USA) and a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive™ MS, Thermo Fisher Scientific GmbH, Bremen, Germany). The browse plant extracts were diluted 10 times and filtered through 0.2 μm

Table 1
Proanthocyanidins and other polyphenol contents of browse plant materials.

Browse plant species name		Proanthocyanidins (mg/g dry matter)				Galloyl derivatives	Quinic acid derivatives	Flavonol aglycones and glycosides (mg/g dry matter) ± SE[c]		
Scientific	Family[a]	Total	Soluble	Bound	Composition[b]	(mg/g dry matter) ± SE[c]		Kaempferol based	Quercetin based	Myricetin based
Euclea racemosa	E	>200	101–150	51–100	PC,PD	2.1 ± 0.1	–	0.3 ± 0.1	1.3 ± 0.1	7.1 ± 0.1
Rhus natalensis	A	>200	>200	101–150	PC,PD	0.2 ± 0.1	2.0 ± 0.1	0.7 ± 0.1	5.8 ± 0.1	1.8 ± 0.1
Maytenus senegalensis	Ce	>200	>200	101–150	PC,PD	<0.1	–	4.7 ± 0.1	4.4 ± 0.1	1.3 ± 0.1
Dichrostachys cinerea	F	101–150	101–150	5–50	PC,PD	6.9 ± 0.1	–	1.4 ± 0.1	7.7 ± 0.1	6.0 ± 0.1
Dodonaea angustifolia	S	51–100	51–100	5–50	PC	0.1 ± 0.1	0.4 ± 0.1	1.1 ± 0.1	1.5 ± 0.1	–
Acacia etbaica	F	5–50	5–50	0–4	PC,PD,PF/PP	0.9 ± 0.1	–	0.7 ± 0.1	5.5 ± 0.1	0.2 ± 0.1
Senna singueana	F	5–50	5–50	–	PC,PD,PF/PP	–	–	9.0 ± 1.6	3.0 ± 0.6	0.2 ± 0.1
Capparis tomentosa	Ca	0–4	–	–	–	–	–	2.1 ± 0.1	2.0 ± 0.1	–
Cadaba farinosa	Ca	–	–	–	–	–	–	–	0.5 ± 0.1	–
Maerua angolensis	Ca	–	–	–	–	–	–	0.4 ± 0.1	0.5 ± 0.1	–

– = not present, / = or.
[a] A = Anacardiaceae, Ca = Capparidaceae, Ce = Celasteraceae, E = Ebenaceae, F=Fabaceae, S = Sapindaceae.
[b] PC = Procyanidins, PD=Prodelphinidins, PF = Profisetinidins, PP = Propelargonidins.
[c] SE = Standard Error.

PTFE syringe filters prior the analysis. The column was an Acquity UPLC® BEH Phenyl (100 × 2.1 mm inner diameter; 1.7 μm; Waters Corporation, Wexford, Ireland). The mobile phase consisted of (A) acetonitrile and (B) 0.1% formic acid. The elution profile was as follows: 0–0.5 min, 0.1% A in B; 0.5–5.0 min, 0.1–30% A in B (linear gradient); 5.0–5.1 min, 30–90% A in B (linear gradient); 5.1–7.1 min, 90% A in B; 7.1–7.2 min, 90–0.1% A in B (linear gradient); 7.2–8.5 min, 0.1% A in B. The injection volume was 5 μl and flow rate 0.5 ml/min. The UV data were collected at 190–500 nm. The heated electrospray ion source (H-ESI II, Thermo Fisher Scientific GmbH, Bremen, Germany) was operated in negative ion mode. The parameters were as follows: spray voltage was set at −3.0 kV, sheath gas (N_2) flow rate at 60 (arbitrary units), aux gas (N_2) flow rate at 20 (arbitrary units), sweep gas flow rate at 0 (arbitrary units), capillary temperature at +380 °C and S-lens RF level at 60. The Orbitrap was set to a resolution of 70,000 and an automatic gain of 3 × 106 was used. Masses were scanned at m/z 150–2000. Pierce ESI Negative Ion Calibration Solution (Thermo Fischer Scientific Inc., Waltham, MA, USA) was used for the calibration. The data was processed with Thermo Xcalibur Qual Browser software (Version 3.0.63, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Other polyphenols were analysed by UPLC-ESI–MS/MS as previously described by Engström et al. (2015), with extracts diluted 10 times and filtered through 0.2 μm PTFE syringe filters prior the analysis.

2.4. Larval exsheathment inhibition assay (LEIA)

The larval exsheathment inhibition assay as described by Jackson and Hoste (2010) was used to measure the anthelmintic properties of the browse plant extracts. Briefly, approximately 1000 ensheathed infective larvae (L_3) from susceptible strains of *H. contortus* were incubated for 3 h in browse plant extracts at concentrations of 1200, 600, 300, 150 μg/ml in PBS, (Phosphate Buffered Saline, 0.1 M phosphate, 0.05 M NaCl, pH 7.2). A negative control (L_3 only in PBS) was added to the assay. Four replicates were included for each dose and for the control. At the end of incubation larvae were washed and centrifuged three times for 3 min at 161g. Finally, the L_3 were submitted to the process of artificial exsheathment by contact with exsheathment fluid (NaClO, 2% w/v; NaCl, 16.5% w/v) after its dilution with PBS (1:400). Then larvae were examined under a microscope at ×200 magnification to identify the proportion of exsheathed L_3 at 0, 20, 40, and 60 min after contact with the exsheathment solution.

Percentage exsheathment was calculated as follows:

$$\text{Exsheathment \%} = \frac{\text{number of exsheathed larvae}}{\text{number of exsheathed larvae} + \text{number of ensheathed larvae}} \times 100$$

PVPP (50 mg/ml PBS) was used (Barrau et al., 2005) to confirm the role of tannins and polyphenols, as PVPP binds to phenolics (Doner et al., 1993), rendering them inactive in the incubation medium. The extracts were incubated at the concentration of 1200 μg/ml PBS with (50 mg) or without PVPP plus a control in four replicates. First, extracts were pre-incubated with PVPP for 3 h and centrifuged at 3,266g for 5 min at 20 °C. Then the supernatant deprived of phenolics was sampled, the precipitate discarded and the supernatant came in contact with L_3.

2.5. Statistical analysis

Data on dose response and polyphenol effect on larval exsheathment were analysed using the PROC MIXED procedure in SAS 9.3 (SAS, 2010). Extract doses were further analysed for orthogonal polynomial contrasts, linear and quadratic. Dose contrasts were unequally spaced, and hence the PROC IML procedure of SAS was used to generate coefficients. The 50% effective extract concentration to inhibit exsheathment (EC_{50}) was calculated using PoloPlus 1.0 (LeOra Software, 2002).

3. Results

3.1. Proanthocyanidins and other polyphenols in browse plant species

PA contents varied among the different browse plant species (Table 1). Higher concentrations of PA with more than 200 mg/g of dry weight were found in *E. racemosa*, *M. senegalensis* and *R. natalensis*. Only traces of PA were found in *C. tomentosa*, while *C. farinosa* and *M. angolensis* did not contain PA. *A. etbaica* contained mainly PC and PD. In addition, MS data supported the presence of oligomeric PP or PF. *D. angustifolia* was found to contain mainly PC. *D. cinerea*, *E. racemosa* and *R. natalensis* contained PC and PD. *M. senegalensis* contained some PC and many oligomeric PD. In proportion, *S. singueana* contained PC and PD but also PP or PF were detected.

Other polyphenols detected in the browse plant species are presented in Table 1. Gallic acid derivatives were detected in six browse plant species with *D. cinerea* having the highest content of 6.9 mg/g of dry weight. Ellagitannins were not detected in any of the browse

Table 2
Dose dependent effect of browse plant extracts on the artificial exsheathment (%) of *Haemonchus contortus* infective larvae (L_3).

Browse plant species	Dose (μg/ml PBS[a])					SEM[b]	*P*-value	
	0	150	300	600	1200		Linear	Quadratic
Euclea racemosa	94.4	24.8	0.0	0.0	0.0	4.09	<0.001	<0.001
Rhus natalensis	99.2	59.8	18.6	0.0	0.0	9.93	<0.001	<0.001
Maytenus senegalensis	91.0	33.6	0.0	0.0	0.0	9.78	0.001	0.001
Dichrostachys cinerea	100.0	33.7	4.3	0.0	0.0	5.01	<0.001	<0.001
Dodonaea angustifolia	100.0	70.2	56.9	10.1	0.0	10.39	<0.001	0.004
Acacia etbaica	98.8	84.1	6.1	1.8	1.2	2.48	<0.001	<0.001
Senna singuenea	100.0	96.9	2.1	0.0	0.0	1.68	<0.001	<0.001
Capparis tomentosa	97.0	89.0	72.3	7.6	0.0	4.07	<0.001	<0.001
Cadaba farinosa	98.0	96.7	97.8	89.9	62.1	4.15	<0.001	0.047
Maerua angolensis	89.1	81.2	50.5	43.9	0.0	9.26	<0.001	0.516

[a] PBS = Phosphate Buffered Saline.
[b] SEM = Standard Error Mean.

Table 3
Extract concentration required to inhibit 50% of L_3 exsheathment (EC_{50}) calculated at 60 min based on the LEIA assay for *Haemonchus contortus* for the ten browse plant extracts.

Browse plant species	Family name	EC_{50}(μg/ml)	Confidence interval (95%)	
			Lower (μg/ml)	Upper(μg/ml)
Euclea racemosa	Ebenaceae	UD[a]	–	–
Maytenus senegalensis	Celasteraceae	UD[a]	–	–
Dichrostachys cinerea	Fabaceae	127.4	83.6	153.8
Rhus natalensis	Anacardiaceae	168.6	97.3	217.4
Senna singuenea	Fabaceae	179.1	159.2	200.5
Dodonaea angustifolia	Sapindaceae	275.9	132.1	425.4
Acacia etbaica	Fabaceae	285.5	229.1	360.3
Capparis tomentosa	Capparidaceae	333.0	289.3	382.6
Maerua angolensis	Capparidaceae	346.1	223.4	497.6
Cadaba farinosa	Capparidaceae	2036.6	1247.2	8342.7

[a] UD = Unable to determine because the EC_{50} values are below the minimal detection limit applied in the assay.

plant species. Quinic acid derivatives were detected in *D. angustifolia* and *R. natalensis*. Kaempferol based flavonols were detected in nine browse plant species and the content was highest (8.9 mg/g of dry weight) in *S. singueana*. Quercetin based flavonols were detected in all browse plant species. The contents varied from 0.5 to 7.7 mg/g of dry weight. Myricetin based flavonols were detected in six samples. The highest contents were found in *E. racemosa* (7.1 mg/g of dry weight) and *D. cinerea* (6.0 mg/g of dry weight).

3.2. Effect of browse plant extract doses on exsheathment and EC_{50} values of extracts

Larval exsheathment was reduced by browse plant extracts and doses (Table 2). There was a linear ($P < 0.001$) decrease in exsheathment after 60 min for all browse plant extracts with increasing doses. The relation between doses and exsheathment was quadratic for all extracts except *M. angolensis*. At the dose of 1200 μg/ml PBS, there was a complete inhibition of exsheathment for all extracts except *C. farinosa* and *A. etbaica* with 62 and 1.2% exsheathment, respectively. At the concentration of 150 μg/ml PBS, the lowest exsheathment was observed in *E. racemosa* and the highest in *S. singuenea*. The highest exsheathment was in *C. farinosa* (86.6%) and the lowest in *E. racemosa* (6.2%), when averaged across doses. The extract dose required to inhibit 50% of the L_3 (EC_{50}) is presented in Table 3. Activity of browse plant extracts on exsheathment was also reflected in the EC_{50} values with the highest EC_{50} in *C. farinosa* and values were below the detection limit for *E. racemosa* and *M. senegalensis*.

3.3. Larval exsheathment of H. contortus with or without polyvinylpolypyrrolidone

The different browse plant extracts (Table 3), were evaluated for the contribution of PA in the inhibition of *H. contortus* exsheathment (Fig. 1) with the exception of *C. farinosa* because of outlying EC_{50} values.

There was a significant ($P \leq 0.001$) difference between control and PVPP treated *A. etbaica*, *C. tomentosa*, *M. angolensis*, *R. natalensis* and *D. cinerea*. No significant ($P > 0.081$) differences were observed between control and PVPP treated *E. racemosa*, *M. senegalensis*, *D. angustifolia*, *S. singueana*.

4. Discussion

The anthelmintic properties of plant polyphenols (Hoste et al., 2015) and their mode of action (Hoste et al., 2012) against nematodes of ruminants have frequently been reported on both tropical and temperate forages. Depending on the plant botanical family, phenological stage and provenance, the type and concentrations of polyphenols vary accordingly. For practical application and wider use, the anthelmintic properties of different forage sources need to be evaluated. In the present experiment, 10 browse species from the semi-arid regions of Ethiopia and browsed by goats were considered. We are not aware of any previous reports on the anthelmintic property in conjunction with polyphenol composition related to these browse species which belong to six different botanical families with Fabaceae and Capparidaceae being the dominant groups. With the exception of *C. farinosa* and *M. angolensis*, the range of browse species selected possess variable concentrations of

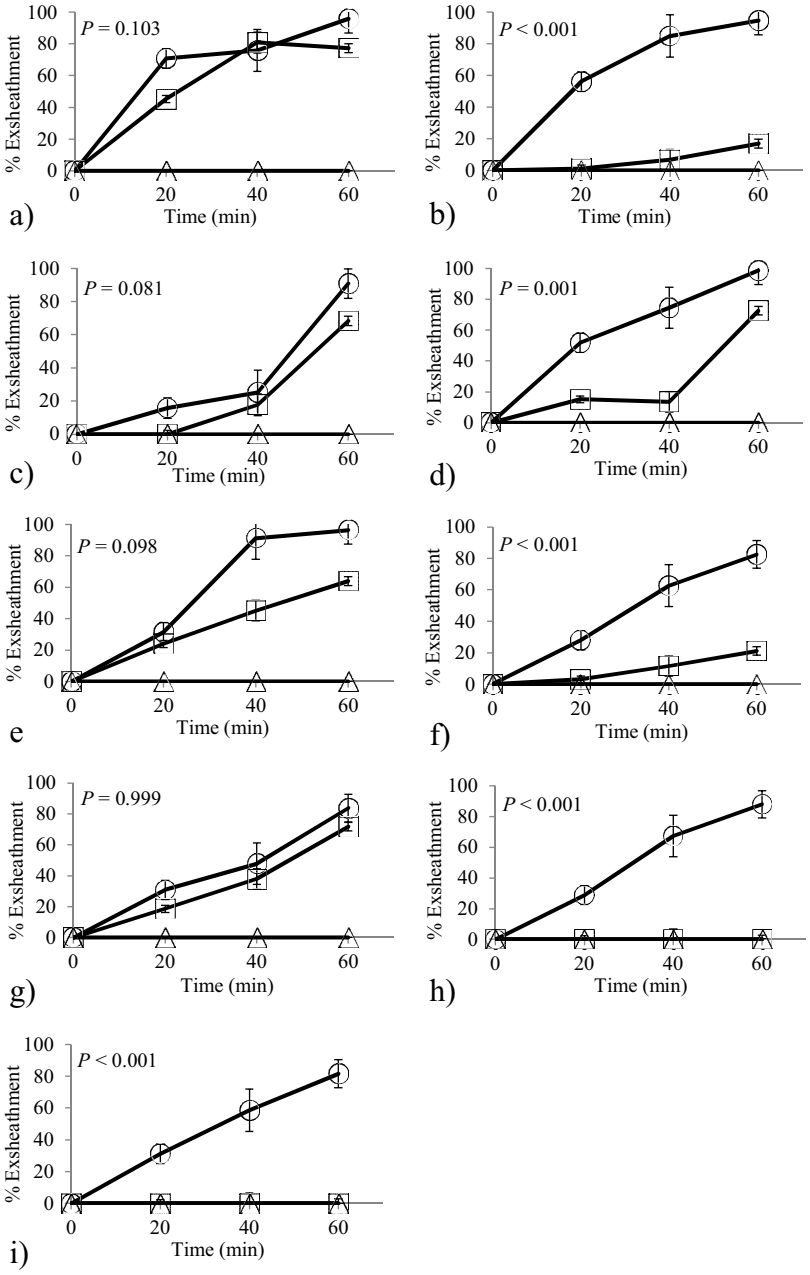

Fig. 1. Condensed tannin and other polyphenols inhibitory activity of browse plant extracts (1200 μg/ml phosphate buffered saline, PBS) in the presence (□) and absence (△) of polyvinylpolypyrrolidone (PVPP) evaluated by comparing *Haemonchus contortus* L_3 exsheathment (mean ± SD, 4 replicates) and control (○, only PBS) at 60 min. P-values indicate the difference between PVPP treated browse plant extract and the control.
(a) *Euclea racemosa*, (b) *Rhus natalensis*, (c) *Maytenus senegalensis*, (d) *Dichrostachys cinerea*, (e) *Dodonaea angustifolia*, (f) *Acacia etbaica*, (g) *Senna singueana*, (h) *Capparis tomentosa*, (i) *M. angolensis*.

PA. Interestingly, PF or PP were found only in the family Fabaceae (*A. etbaica* and *S. singueana*). Galloyl derivatives were detected in all except *S. singueana* and the capparidaceae family. Kaempferol derivatives were present in all browse except *C. farinosa*, while quercetin based polyphenols were present in all browse. Myricetin based polyphenols were absent in *D. angustifolia* and Capparidaceae family.

The first objective of the present study was to evaluate the anthelmintic properties of the 10 browse species using a LEIA. The latter assay was chosen because of its simplicity, low cost, sensitivity, reproducibility and it allows the activity of tannin and/or flavonoids to be determined (Alonso-Díaz et al., 2011). Besides, the exsheathment of the L_3 in *H. contortus* represent the transition from the free-living to the parasitic stage (Bahuaud et al., 2006; Brunet et al., 2007) and the LEIA examines inhibitory activity at the early stage of the parasite life cycle. More than 89% of the L_3 exsheathed in the control group after 60 min. At the concentration of 1200 μg/ml, with the exception of *C. farinosa*, exsheathment was totally blocked in all extracts regardless of PA concentration. Inhibition was observed at the lowest concentration (150 μg/ml), and the highest inhibition for *H. contortus* appeared to be related to the highest PA concentration (>200 mg/g). But at the concentration of 150 μg/ml, more sever inhibition in *D. cinerea* (PA, 101–150 mg/g) compared to *R. natalensis* (>200 mg/g) could be related to its high content of galloyl derivatives or PA composition. High doses (1200 μg/ml) of tropical tanniniferous browse

extracts inhibited *T. colubriformis* L_3 exsheathment in another study (Alonso-Díaz et al., 2008).

With the exception of *D. angustifolia*, PA containing extracts severely inhibited exsheathment at 300 μg/ml. The lower inhibition in *D. angustifolia* could be due to the absence of PD in the PA. More potent inhibition of PA on the exsheathment in *H. contortus* and *T. colubriformis in vitro* were observed with a higher PD:PC ratio (Brunet and Hoste, 2006; Quijada et al., 2015). In *C. tomentosa* with the lowest PA concentration (0–4%) and *M. angolensis* lacking PA, L_3 exsheathment was blocked at 1200 μg/ml suggesting browse plant compounds other than PA are involved in the inhibition of L_3 exsheathment. The EC_{50} values, in general, corresponded with PA in the same direction. *E. racemosa* and *M. senegalensis* with the highest PA concentration (>20%) had the lowest EC_{50} values, below the detection limit. The EC_{50} values for the PA containing extracts were, on average, lower than the values reported by Moreno-Gonzalo et al. (2013) for heather species in *H. contortus* L_3 exsheathment *in vitro*. Interspecies differences, polyphenol concentration and composition and the strain of *H. contortus* could explain the difference.

The second objective of the present study was to examine the role of PA and other polyphenols in the inhibition of L_3, using PVPP. The use of PVPP aimed at inactivating tannins and flavonol glycosides (Doner et al., 1993; Alonso-Díaz et al., 2008). Based on the EC_{50} value, *C. farinosa* was far less potent compared to other extracts and, therefore, excluded from the PVPP assay. There was a complete inhibition of exsheathment with and without PVPP addition for *C. tomentosa* and *M. angolensis*. However, PA are only present in small quantities in *C. tomentosa* and absent in *M. angolensis*, while none of these two extracts possess galloyl derivatives indicating PA and other polyphenols were not involved in the inhibition of L_3 exsheathment. Therefore, anthelmintic activities in the two species could be due to the presence of PSM other than PA and phenols. A partial inhibition in *A. etbaica*, *D. cinerea* and *R. natalensis* indicate that PA and flavonols were partly responsible for the inhibition, but also other biochemical components could have blocked exsheathment (Azando et al., 2011). Moreover, Alonso-Díaz et al. (2008) noted that, in addition to CT, flavonoid glycosides, other tannins and/or polyphenols can have additional anthelmintic effect in tropical browse. The partial inhibition, therefore, could be due to a higher proportion of polyphenol aglycones affecting the binding activity of PVPP. Polyvinylpolypyrrolidone preferentially binds to polyphenol aglycones than polyphenol glycosides (Laborde et al., 2006). The absence of a significant difference between PVPP treated *E. racemosa M. senegalensis* and *S. singueana* suggests, the anthelmintic effect was mainly due to PA and other polyphenols related to the presence of kaempferol, quercetin and myricetin based flavonol glycosides.

The third objective was to assess the relationship between polyphenol composition, concentration and exsheathment. All PA containing (≥5 mg/g) extracts, inhibited L_3 exsheathment and confirmed the anthelmintic properties of PA on *H. contortus* in other PA containing forages *e.g.* sainfoin extract *in vitro* (Brunet et al., 2007) and *in vivo* with quebracho extracts administration to goats (Paolini et al., 2003). One common characteristics of all PA containing extracts in the present experiment was the presence of PD, and they possibly are the main contributor of the anthelmintic effect. The anthelmintic activity of PD was identified in *in vitro* assays using different monomeric units of PA. Brunet and Hoste (2006) used flavan-3-ols and their galloyl derivatives in a LEIA and found that PD monomers were most responsible in reducing *H. contortus* L_3 exsheathment. A similar observation was associated with the anthelmintic property of PA in *H. contortus* and *T. colubriformis* with PD monomers (Quijada et al., 2015).

Although *E. racemosa*, *R. natalensis* and *M. senegalensis* had a similar PA concentration, persistence of exsheathment in the PVPP treated *R. natalensis* appeared to be due to its content of quinic acid derivatives, which is absent in the other two species or the presence of other non-phenolic compounds. Klongsiriwet et al. (2015) reported a synergistic anthelmintic effect between tannins and quercetin, against *H. contortus* larval exsheathment. Similarly, synergy could contribute to the anthelmintic property in *R. natalensis*. Despite the relatively lower PA concentration compared to the above three species, *D. cinerea* had a lower EC_{50} and showed a partial inhibition upon PVPP addition. This could be due to its higher concentration of quercetin based flavonol glycosides. Barrau et al. (2005) previously observed that at high concentration of 1200 μg/ml, quercetin-3-rutinoside had anthelmintic effect on *H. contortus* larval migration *in vitro*. It could also be related to the high concentrations of galloyl derivatives and myricetin based flavonol glycosides. *S. singueana*, with a lower PA content but a high content of kaempferol based flavonol glycosides, showed higher inhibition with a lower EC_{50} value. This could be mainly due to the content of kaempferol based flavonol aglycones and glycosides, and Barrau et al. (2005) observed the anthelmintic effect of kaempferol-3-rutinoside at high concentration. *A. etbaica* had the highest EC_{50} (285.5 μg/ml) values and was a relatively less potent extract among the PA containing extracts.

Comparing the botanical families in the present experiment, Ebenacae, Fabaceae, Celasteracea, commonly contain PA and with the exception of *A. etbaica,* their anthelmintic property mainly originates from PA and polyphenol contents. In Sapindaceae (*D. angustifolia*), inhibition of exsheathment exist mainly due to PA, while, in the Capparidaceae family other plant secondary metabolites exert anthelmintic property. Larvicidal activities of some selected Anacardiaceae, Fabaceae and Ebenaceae plant families have been reported previously with *H. contortus* (Diehl et al., 2004), in agreement with results presented here.

The present study dealt with only one nematode species (*H. contortus*), one type of *in vitro* assay and one extract from each browse plant species. Besides, as *in vitro* systems do not completely simulate conditions in the gastrointestinal tract or take into account, for example feed intake fluctuations or the physiological state of the animal, care should be taken to translate the data of the present study to *in vivo* efficacy of the browse plant species. However, the present study provides an indication for the potential of several browse plant species as anthelmintic agents against *H. contortus in vivo*. CT from sainfoin exhibited anthelmintic properties against exsheathment of *H. contortus in vitro* and *in vivo* in sheep (Brunet et al., 2007). The browse plant species used in the present study have been reported to be voluntarily consumed by goats (Yayneshet et al., 2008; Mengistu et al., 2016) and at optimal intake, may exhibit anthelmintic activity against *H. contortus*. Anthelmintic properties of the browse plant species, however, have to be examined under practical farming conditions before foreseeable future practical application to control *H. contortus* to improve productivity and health of goats in the study area and for wider application in similar regions.

5. Conclusions

Browse plant extracts showed anthelmintic activity against *H. contortus* L_3 exsheathment, attributable mainly to the PA contents and also the PD composition. The presence of other polyphenols such as kaempferol, quercetin and myricetin based flavonol glycosides also appeared to contribute to the anthelmintic property for most browse plant species. However, the possible inhibition of exsheathment by other non-phenolic constituents was apparent as evidenced in browse plant species with negligible polyphenol contents, *e.g. C. tomentosa* and *M. angolensis*. In comparison, extracts of *E. racemosa* and *M. senegalensis* are highly potent inhibitors

of L_3 exsheathment. Overall, the results confirmed that PA, other polyphenols and non-phenol components in browse plant species may act in consortium to exert anthelmintic effect on *H. contortus*.

Acknowledgements

This work was supported by the Netherlands Organization for International Cooperation in Higher Education, Wageningen Institute of Animal Sciences, Wageningen University & Research, the Netherlands, LegumePlus Marie Curie Initial Training Network, INRA, Toulouse, France, University of Turku, Finland. The authors acknowledge facilities provided by Mekelle University, Ethiopia, College of Veterinary Medicine & Agriculture and Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Ethiopia. Chemical analyses on the UHPLC-DAD-ESI–MS/MS system were made possible by a Strategic Research Grant of the University of Turku (Ecological Interactions). The authors thank Atte Tuominen for his help in the UPLC-DAD-ESI–MS/MS analysis.

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